Detection of prions in blood from patients with variant Creutzfeldt-Jakob disease

Highly Sensitive and Specific Detection of Prions in Blood of Variant Creutzfeldt-Jakob Disease Patients

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ABSTRACT

Human prion diseases are infectious and invariably fatal neurodegenerative diseases, including sporadic Creutzfeldt-Jakob disease (sCJD), the most common form, and variant CJD (vCJD) which is caused by interspecies transmission of prions from cattle infected by bovine spongiform encephalopathy. Development of a biochemical assay for the sensitive, specific, early and non-invasive detection of prions in blood of patients affected by prion disease is a top medical priority to increase the safety of the blood supply. vCJD has already been transmitted from human-to-human by blood transfusion and the number of asymptomatic carriers of vCJD in the UK alone is estimated to be 1 in 2,000 people. In this study we used the protein misfolding cyclic amplification (PMCA) technique to analyze blood samples from 14 cases of vCJD and 153 controls, including patients affected by sCJD, other neurodegenerative or neurological disorders as well as healthy subjects. Our results show that we can detect PrP^{Sc} with 100% sensitivity and specificity in the tested vCJD samples. Detection was possible in any of the blood fractions analyzed and can be done with as little as a few µL of sample volume. The PrP^{Sc} concentration in blood was estimated to be ~0.5 pg/mL. Our findings suggest that PMCA may be useful for pre-mortem non-invasive diagnosis of vCJD and to identify prion contamination of the blood supply. Further studies are needed to fully validate the technology and investigate the earliest time during the pre-clinical phase of vCJD when PrP^{Sc} is detectable in blood.

Human prion diseases are infectious and invariably fatal neurodegenerative diseases, including sporadic Creutzfeldt-Jakob disease (sCJD), the most common form, and variant CJD (vCJD), which is associated with the consumption of cattle infected with bovine spongiform encephalopathy (*1, 2*). Currently there is not a regulatory approved assay for sensitive, objective and non-invasive biochemical diagnosis of these diseases. This is a major problem for public health, because prion diseases are known to be transmitted iatrogenically between human-to-human and because asymptomatic carriers may far outnumber the clinically affected individuals, due to the long pre-symptomatic stage of the disease, which may span several decades (*3*).

The infectious agent responsible for these diseases, termed prion, appears to be composed exclusively of a conformationally altered form of a naturally occurring protein, named PrP^{Sc}, which has the exceptional ability to infect individuals and propagate in the body without the need for genetic material (*4*). PrP^{Sc} is not only the main component of the infectious agent and the likely culprit of neurodegeneration, but is also the best surrogate marker for the disease. A major challenge for early diagnosis based on PrP^{Sc} detection is that this marker is present at high levels only in the CNS at late stages of the disease. However, several lines of evidence indicate that prions are also present in small quantities in peripheral tissues and biological fluids, such as lymphoid organs, CSF and blood (*5, 6*). Detection of PrP^{Sc} in blood is very challenging as little is known about its quantity, nature and distribution in this fluid. Based on animal infectivity studies it is estimated that in rodent plasma and buffy coat fractions there is as little as 1-10 LD_{50} infectious units in 1 mL of whole blood, which translates into the equivalent to a 10^{-7} - 10^{-9} dilution of the brain (*7*). Moreover, both high levels of normal PrP^{C} found in these

fluids as well as uncertainty on the biochemical and structural properties of blood-derived PrP^{Sc} (*8-12*), would make it very difficult to develop a diagnostic test relying on existing biochemical and immunological methods for detecting PrP^{Sc}. In the case of CJD it is also imperative that an effective test must not only have high sensitivity but also be extremely specific. Considering that no treatment is available for this disease, it is not ethically acceptable to have a test with high frequency of false positives.

Our strategy to achieve sensitive and specific detection of PrP^{Sc} is to use an amplification technology that reproduces PrP^{Sc} replication *in vitro* (*13*). This system is called PMCA (Protein Misfolding Cyclic Amplification) and consists of cycles of accelerated prion replication combining phases of PrP^{Sc} growing with fragmentation of the polymers to increase the number of seeding-competent units. The cyclic nature of the system permits employing as many cycles as required to reach the amplification state needed for the detection of PrP^{Sc} in a particular sample (*13, 14*). We have previously reported proof-of-concept experiments in which the technology was applied to replicate the misfolded protein from diverse species (*15*). The technology has been automated, leading to a dramatic increase on efficiency of amplification. In its most current form in vCJD samples (*16*), one round of 96 PMCA cycles (2 days) results in the ability to detect up to a 100 million-fold (10^8) dilution of brain, whereas after 2 rounds we reached the highest detectability possible, which is around 10 billion-fold (10^{10}) dilution. Moreover, our results demonstrate that PMCA is capable of detecting as little as approximately 26 monomers of PrP (*17, 18*), which according to recent data on the minimal size of the infectious particle (*19*), would correspond to a single particle of oligomeric infectious PrP^{Sc}. These data demonstrates that PMCA has a similar power of

amplification as PCR techniques used to amplify DNA and opens great possibilities for development of a highly sensitive detection of PrP^{Sc}. Indeed, we have demonstrated that after amplification we can detect PrP^{Sc} in blood of hamsters experimentally infected with scrapie during both the symptomatic (*20*) and pre-symptomatic phases of the disease (*21*) as well as in urine of humans affected by vCJD (*16*). A recent report showed PrP^{Sc} detection by PMCA in white blood cells of few patients affected by vCJD (*22*). Using an ovine PrP^{C} substrate for the PMCA reaction, this study showed a limit of detection equivalent to 10^{-7} brain dilution and positive PrP^{Sc} detection in 3 of the 4 vCJD samples tested, suggesting the possibility of the absence of prionemia in certain patients (*22*). The major goal of our study was to develop a more sensitive PMCA assay for PrP^{Sc} detection in vCJD samples and use it to evaluate the presence of prions in blood from patients affected by this disease, estimate sensitivity and specificity as well as the approximate quantity of PrP^{Sc} present in vCJD blood.

RESULTS

To begin analyzing the possibility to detect vCJD PrP^{Sc} in blood by PMCA, we first performed spiking experiments diluting vCJD brain homogenate into healthy whole blood in order to optimize conditions and evaluate the limit of sensitivity. As observed before in our experiments to detect PrP^{Sc} in animal blood, this fluid drastically inhibited PMCA reaction (*20, 21*). For this reason, it is necessary to process the blood samples to enrich in PrP^{Sc} and remove other blood components that interfere with PMCA. The process consists of a centrifugation in the presence of sarkosyl, followed by washing in PBS (Fig. 1). The resulting material was subjected to sequential rounds of PMCA using

a substrate of transgenic mice Tg6816 brain homogenate expressing human PrP with Met/Met genotype at position 129 (TgHuPrP(129MM)). After the first round of PMCA we can detect up to a 10^{-6} final dilution of vCJD brain homogenate spiked in whole blood (Fig. 2). This level of detection is clearly lower than the one observed when vCJD brain homogenate was diluted directly in conversion buffer (*16*), suggesting that even despite the cleaning procedure, there is still some interference of blood components. After 2 rounds of PMCA we can detect PrP^{Sc} up to a 10^{-9} dilution and after 3 rounds we reach the maximum level of detection, equivalent to a 10^{-10} dilution of the brain (Fig. 2).

We then analyzed blood from 14 patients suffering from vCJD and compared it to samples from people affected by other neurodegenerative (62 samples) or non-degenerative neurological disorders (26 samples) and healthy individuals (49 samples) (Table 1). Our results show that most of vCJD blood samples analyzed were positive after 2 rounds of PMCA and all after 3 rounds, whereas none of the control samples gave any signal, even after 5 rounds of PMCA (Fig. 3, Table 1). These data indicate that the PMCA technology has a 100% sensitivity (95% confidence interval 76.8-100%) and specificity (95% confidence interval 97.6-100%) for detection of PrP^{Sc} in blood of vCJD patients. To analyze whether detection of PrP^{Sc} in blood is specific for vCJD compared to other forms of human prion diseases, we studied samples from sCJD blood. The results showed that none of the 6 samples tested of sCJD whole blood was positive for PMCA even after 5 rounds of amplification (Fig. 3). These later results do not necessarily mean that there is not PrP^{Sc} in sCJD blood, but that with this set of PMCA conditions it is not detectable. Indeed, a spiking experiment in which healthy blood containing different dilutions of sCJD brain homogenate showed no detection of PrP^{Sc}

even at the lowest dilutions used (Fig. S1). We are currently optimizing a different set of PMCA conditions to efficiently detect sCJD prions.

To analyze the blood fraction that carries PrP^{Sc}, we obtained plasma and white cells from 2 additional cases of vCJD. PrP^{Sc} was detectable in both of these fractions after a similar number of amplification cycles (Fig. 4), indicating that the amount of prions in plasma and cells is similar. However, we cannot rule out substantial differences in the quantity of PrP^{Sc} in different blood fractions that were masked by the very high efficiency of the assay. Also, PrP^{Sc} was detectable in whole blood from a patient collected at two different times during the symptomatic disease (Fig. 4 lanes 5 and 6). We analyzed similar fractions of sCJD blood, confirming the absence of prions able to replicate in the setting used to detect vCJD prions (Fig. 4B).

We also analyzed the molecular typing of prions amplified from blood by PMCA (Fig. S2). As expected, the material amplified from blood has the same type 2B pattern displayed by prions from vCJD brain homogenate, in contrast to the type 1 and 2 patterns of classical sCJD.

To estimate the minimum volume of blood required for our assay, we tested different quantities of whole blood from 2 different vCJD patients. Our results show that as little as 3 or even 0.3 µl of blood are sufficient to readily detect PrP^{Sc} in human vCJD blood after PMCA amplification (Fig. 5A). The differences in the minimum volume of blood needed to detect the signal for the 2 distinct samples tested likely reflect the dissimilar amounts of prions present in different blood samples. However, we cannot rule out that some samples may have also different amounts of blood components that interfere with the PMCA reaction. Reduction of the volume of material used also avoided the need of

a pre-cleaning step, since the concentration of PMCA inhibitors present in blood is reduced to a level that does not significantly interfere with the reaction. Indeed, in samples of healthy blood spiked with a 10^{-8} dilution of vCJD brain homogenate, the signal in the second PMCA round was higher when the volume of sample was lower (Fig. 5A). Elimination of the pre-cleaning step is important for practical and routine use of the technology in blood detection since this step is time- and effort-demanding. The small minimum volume of blood needed for PrP^{Sc} detection added to the known ability of PrP^{Sc} to bind to a variety of surfaces (*23-25*), led us to hypothesize that just by placing a blood sample in the PMCA tube, maybe enough prions would bind to the tube and enable prion replication in a PMCA reaction. To investigate this possibility, 100 µl of whole blood from 2 different patients affected by vCJD, as well as 5 healthy controls and normal blood spiked with a 10^{-5} and a 10^{-9} final dilution of vCJD brain homogenate were incubated in PMCA tubes for 1h at room temperature. Thereafter, the entire volume of the samples was removed and 100 µl of PMCA substrate (10% TgHuPrP(129MM)) was added to the same tube. After serial rounds of PMCA the presence of PrP^{Sc} was detected by western blots after PK digestion. The results clearly showed that a PrP^{Sc} signal was detected in both vCJD blood samples, as well as in the positive controls spiked with vCJD brain homogenate (Fig. 5B). Interestingly, the round in which the samples of vCJD blood were detected after tube binding of the agent was the same as by using the standard protocol of centrifugation in detergent. Indeed, sample vCJD4 was detectable in both cases in round 2 and sample vCJD7 was detectable after 3 rounds of PMCA (compare Fig. 3 and 5B). No signal was detectable in any of the healthy controls. These results may enable the implementation of a simpler

procedure for PrP^{Sc} detection in blood, leading to substantial savings of time and samples, but the robustness of the assay need to be confirmed by analyzing larger number of vCJD blood samples. The efficient detection of PrP^{Sc} in tubes that were simply exposed to the infectious material shows once again the similar power of amplification of PMCA compared to PCR reactions.

Finally, to estimate the quantity of PrP^{Sc} present in vCJD blood, we used the quantitative PMCA technology which compares the number of cycles required to detect the signal with those of blood spiked with known concentrations of PrP^{Sc} (*18*). Our data showed that 11 of the 14 samples analyzed were clearly detectable after 2 PMCA rounds (Figs. 3 and 4). By comparison with the spiked samples we estimate this to be equivalent to the amount of PrP^{Sc} present in a 10^{-9} dilution of the brain which extrapolates to about $5x10^{-13}$ g/ml. This concentration is similar to previous estimations using bioassays which indicated that the quantity of prions in blood is in the order of 1-10 LD_{50} per ml of blood (*7*). Considering our previous estimation of the concentration of PrP^{Sc} excreted in urine of vCJD patients (*16*), we conclude that the concentration in blood is between 2 and 3 orders of magnitude higher than in urine.

DISCUSSION

So far, 229 cases of vCJD have been reported mostly in the UK and France (with 4 cases in USA) and the future of this epidemic remains unknown. Fortunately, the spreading of classical BSE has been largely controlled, thanks to the implementation of feeding restrictions and surveillance (*26-28*). However, the appearance of atypical and genetic forms of BSE is an additional concern as the characteristics of transmission of

these new forms may be different from traditional BSE (*29-32*). Nevertheless, it is estimated that millions of people have been exposed to BSE prions and it is currently unclear how many people may silently carry infectious material. Indeed, studies in transgenic mice models of human prion disease showed that infection with BSE prions frequently produces subclinical or carrier states, which upon a secondary infection can produce full-blown disease (*3, 33, 34*). Strikingly, a recent study searching for PrP^{Sc} immunoreactivity in archived surgically resected appendixes in the UK estimated that 30,000 people in this country are asymptomatic carriers of vCJD infection (*35*). It is probable that this number is underestimated, since the methods used to detect prions in lymphoreticular specimens are unlikely to have 100% sensitivity. The possible existence of a large number of carriers of vCJD prions represents a significant risk for iatrogenic transmission of vCJD from human to human, a pathway that has already been shown to happen in other human prion diseases such as kuru and iatrogenic CJD (*36*). Iatrogenic transmission of vCJD through blood transfusion is perhaps the most significant concern because blood transfusion is a fairly common practice in medicine. Unfortunately this route has already proven to occur, since several cases have been linked to transfusion of blood donated by infected individuals at the pre-clinical stage of the disease (*37-39*). Although it is not known for certain that all subclinically infected individuals are able to infect others by means of blood transfusion, it is reasonable to assume this possibility as a precautionary principle.

Currently there is no pre-mortem biochemical diagnosis for vCJD or any validated procedure to detect prions in blood or other human derived materials that might represent a concern for iatrogenic transmission of vCJD. Upon the onset of the first

clinical abnormalities, people affected by vCJD die at an average of 2 years. The disease normally starts with psychiatric alterations (depression, anxiety, hallucinations) which are fairly common in other diseases and only begin to show more typical signs of a neurodegenerative condition (ataxia, myoclonus, dementia) several months after the first abnormalities. Even at this time the diagnosis of vCJD is very uncertain and is only confirmed by postmortem examination of the brain for the presence of prions with the vCJD signature and the associated brain abnormalities. Having a definitive diagnosis of vCJD at the early stages of the clinical disease is of utmost importance for differential diagnosis. Furthermore, early diagnosis of vCJD would allow any potential therapy to be given as soon as possible before substantial brain damage, which has been a problem with clinical trials in this group of diseases. It would also allow any public health measure to be implemented as soon as possible, e.g. tracing of any recipients of blood donated by an individual patient.

Exciting recent progress in high sensitive detection of prions in biological fluids has been reported with the use of prion amplification techniques, including PMCA and RT-QuIC (*40, 41*). Both PMCA and RT-QuIC take advantage of the prion conversion ability by seeding of protein misfolding to substantially amplify the signal and detect small amounts of PrP^{Sc}. Detection of PrP^{Sc} by RT-QuIC in cerebrospinal fluid (*42, 43*) and nasal brushings (*44*) is currently being employed in clinical diagnosis of sCJD. Unfortunately, much less work has been done with the application of RT-QuIC to vCJD and blood samples. Our current study demonstrates that small quantities of PrP^{Sc} are circulating in blood (both in plasma and associated with white cells) of all vCJD patients analyzed. These particles can be readily detectable by PMCA with 100% sensitivity and

specificity. It is important to highlight the caveat that these estimations are based on a small number of vCJD blood samples analyzed (14 cases). Future double-blind studies are needed with a larger number of samples to allow statistical validation of the test. Nevertheless, although 14 samples may look little, it actually represents 6.1% of all the cases reported in the world since the appearance of vCJD more than 10 years ago. Furthermore, for many of the patients, samples were not collected or stored, and for many others the samples have been sequestered by regulatory health authorities to be used for validating potential commercial blood detection assays.

Several pieces of evidence indicate that PrP^{Sc} might be present in blood of people infected, but not yet exhibiting the clinical symptoms of the disease. Indeed, various cases of vCJD were acquired by transfusion with blood taken from donors at the pre-clinical stage of the disease (*37-39*). Since, PMCA has the capability to detect the equivalent of a single particle of PrP^{Sc} (*17, 18*), our findings suggest that PMCA may be useful as a pre-symptomatic non-invasive diagnosis of this disease. Supporting this conclusion, we have previously shown pre-clinical detection of prions in blood samples of animals experimentally infected with prion diseases (*21*). Also, and perhaps more importantly PMCA might be useful to identify prion contamination of the blood supply, which will minimize the iatrogenic spreading of this disease. Further studies need to be done to demonstrate the usefulness of PMCA for pre-symptomatic detection of vCJD prions and to investigate the earliest time during the pre-clinical phase of vCJD in which PrP^{Sc} is detectable in blood.

In summary, our findings indicate that PMCA offers a great opportunity to detect PrP^{Sc} in blood of people infected by vCJD. This technology may have multiple practical

applications, including disease diagnosis, blood banks safety, estimation of the possible pool of vCJD infection in the population, etc. The main limitations of our current study are the small number of samples analyzed, the fact that all samples were coming from people at later stages of the clinical disease and the lack of studies with blood samples from pre-symptomatic cases. The later is a very difficult task, since at this time it is not possible to identify people that have been infected with vCJD before the clinical symptoms of the disease are evident. Studies in non-human primates which have been experimentally infected with vCJD and blood samples collected longitudinally at various time points during the incubation period, offer the best alternative to analyze this possibility.

METHODS

Study design

For this study we utilized blood samples from 14 vCJD patients and 153 controls, distributed as indicated below. Samples were obtained randomly without any exclusion or inclusion criteria other than the respective clinical diagnosis. Data reported included all samples analyzed. No samples or data were excluded after analysis. Although this study was not done with blinded samples, we analyzed randomly many vCJD samples and controls at the same time, from initial centrifugation until western blot. The identity of the samples was seen only after the results were obtained.

Patients samples

Samples of frozen blood were collected at different stages of clinically diagnosed vCJD from 14 different patients. Twelve of the samples were collected in the UK and 2 of

them in Italy. The disease was confirmed post-mortem by neuropathological and biochemical analysis. As controls we used four groups of whole blood samples, including 6 sCJD patients, 62 patients affected by other neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and lewy bodies dementia), 26 patients affected by non-degenerative neurological disorders (vascular dementia, traumatic brain injury, stroke, epilepsy, encephalitis, and mood disorders) and 49 healthy individuals (Table 1). Additionally, we analyzed 5 samples of plasma and 5 white blood cells coming from different sCJD patients, adding up to 16 the total number of samples analyzed from sCJD. The diagnosis of sCJD was also confirmed post-mortem by western blot and neuropathological analyses, while the diagnosis of other neurological diseases was determined clinically with the help of imaging and biochemical assays when available. Blood collection was approved by the respective Institutional Review Boards at the authors' centers.

Processing of blood samples

Samples of whole blood, and in some cases separated plasma or white cell fractions, were centrifuged to remove the bulk of proteins and other components that interfered with the PMCA reaction (Fig. 1). Briefly, 250 µl of sample was mixed and incubated with 1 volume of 20% sarkosyl for 10 min at room temperature. Thereafter, samples were centrifuged at 100,000 x g during 1h at 4°C, supernatant was discarded and pellet washed in 250 µl of PBS. Tubes were centrifuged again at 100,000 x g for 30 mins at 4°C. Pellet was resuspended directly in 10% brain homogenates from TgHuPrP(129MM), prepared as described below.

PMCA procedure

The PMCA reaction was carried out as previously described (*16, 45*), using as substrate brain homogenate from transgenic mice expressing human PrP with Met/Met genotype at position 129 (TgHuPrP(129MM)). These animals were kindly provided by Dr. Glenn Telling (Colorado State University). Brain substrate was prepared at a concentration of 10% (weight/volume) in conversion buffer (PBS supplemented with 150 mM NaCl, 6mM EDTA, 0.05% digitonin and 1% Triton X-100) with protease inhibitors (Complete mini EDTA-free, Roche). Debris were removed by a low speed centrifugation (800 x g, 1 min, 4°C) and brain homogenates were stored frozen at -80°C until further use.

For PMCA, samples were subjected to a first round of 144 cycles of PMCA in 0.2 ml tubes (Eppendorf, cat. N. 951010022) containing 3 teflon beads (Hoover precision products). Each cycle consisted of 29 min and 30 s of incubation at 37/40°C followed by a 30 s pulse of sonication set at an amplitude of 30, using Qsonica microsonicator (Model Q700) equipped with a titanium horn. Subsequent rounds of 96 PMCA cycles were done by taking an aliquot of the amplified material which was diluted 10-fold into fresh TgHuPrP129MM brain homogenate. No multichannel pipette was used, to reduce risk of cross contamination. After each round of PMCA, samples were taken for detection of PrP^{Sc} using Western blot after digestion with proteinase K, as described (*16, 45*).

Figure legends

Figure 1. Schematic representation of the blood processing and PMCA procedure. To remove inhibitors of PMCA reaction, samples of whole blood (or separated blood components) were incubated with 1 volume of 20% sarkosyl. After high speed centrifugation, pellets were washed in PBS and centrifuged again. The new pellets were resuspended directly in 10% TgHuPrP(129MM) and placed in a 0.2 ml tube with 3 teflon beads. Samples were subjected to a first round of 144 PMCA cycles, followed by subsequent rounds of 96 PMCA cycles. PrP^{Sc} signal was detected by Western blots after proteinase K digestion.

Figure 2. Optimization and limit of detection of vCJD PrP^{Sc} in blood. In order to optimize PrP^{Sc} detection by PMCA and determine the limit of detection, whole blood from a healthy person was spiked with vCJD brain homogenate at distinct final dilutions (10^{-4} to 10^{-11}). After processing by high-speed centrifugation in the presence of sarkosyl (as described in Fig. 1), samples were subjected to various rounds of PMCA. The PrP^{Sc} signal was assessed by Western blot analysis after PK digestion. N refers to the normal (healthy) brain homogenate, used as migration control marker. Numbers in the left indicate the position of molecular weight markers.

Figure 3. PrP^{Sc} detection in vCJD blood using PMCA. Representative samples of whole blood (250 µL) from 12 patients affected by vCJD, 5 healthy controls, 15 patients affected by other neurodegenerative and neurological disorders and 6 sCJD patients were subjected to 5 rounds of PMCA, as described in Methods. PrP^{Sc} signal was detected by Western blot after PK treatment. N refers to the transgenic mice normal brain homogenate without PK treatment used as migration control. Numbers in the left indicate the position of molecular weight markers.

Figure 4. Detection of PrP^{Sc} in different blood fractions and at distinct times during the clinical disease. **A.** Samples of whole blood (WB), plasma (PL) and white blood cells (WBC) from 2 patients (#13 and #14) affected by vCJD and one healthy control were tested to analyze the presence of PrP^{Sc} in different blood fractions. Also whole blood from patient #13 was collected and tested at two different times during the clinical phase of the disease. Samples were processed and subjected to rounds of PMCA as described in Methods and Fig 1. The content of each lane is: 1, vCJD patient #13 plasma; 2, vCJD patient #14 plasma; 3, vCJD patient #14 WBC; 4, healthy control plasma; 5, vCJD patient #13 whole blood "a" (collected on 09/2002); 6, vCJD patient #13 whole blood "b" (collected on 11/2002); 7, healthy control WBC; 8, healthy control whole blood; 9, molecular weight standard. **B.** Samples of plasma (5) and WBC (5) from 10 confirmed sCJD patients were centrifuged after incubation with sarkozyl and analyzed by a first round of 144 cycles of PMCA, followed by 4 rounds of 96 cycles each. In both panels, N refers to the transgenic mice normal brain homogenate without PK treatment used as migration control. Numbers in the left indicate the position of molecular weight markers

Figure 5. Detection of PrP^{Sc} in small volume of blood and removal of pre-cleaning step. A. To estimate the minimum volume of whole needed for blood detection, different volume of whole blood from 2 vCJD patients (samples #4 and 6) were directly added to a 10% brain homogenate from TgHuPrP(129MM). As positive controls we used healthy blood spiked with a 10^{-8} dilution of vCJD brain. Samples were subjected to 3 sequential rounds of PMCA and PrP^{Sc} detected by Western blot. **B**. Samples of whole blood (100 µl) from 2 vCJD patients (#4 and 7), 5 healthy controls and two blood

samples from controls spiked with 10^{-5} or 10^{-9} dilutions of vCJD brain homogenate were incubated with PMCA tubes containing 3 teflon beads for 1h at room temperature in an end-over-end mixer. The content was removed and 100 µL of PMCA substrate were added to the tube. Samples were subjected to various rounds of PMCA cycles and PrP^{Sc} was detected by Western blot after PK digestion. In both panels, N refers to the transgenic mice normal brain homogenate without PK treatment used as migration control and the numbers in the left indicate the position of molecular weight markers.

Supplementary Materials

Supplementary figure 1. Spiking of sCJD brain homogenate in blood.

Supplementary figure 2. Molecular typing of vCJD prions amplified by PMCA from blood.

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Author Contributions: L.C.-M. designed the studies, carried out the majority of the experiments, analyzed the results and prepared the figures. F.M. and S.P. performed PMCA analysis of some of the patients. J.I., F.T. and P.S. provided blood samples and critically reviewed the manuscript. C.S. is the principal investigator on the project and was responsible for coordinating research activity, analyzing the data, funding, writing the manuscript and producing the final version of the article.

Competing Interest: Dr. Soto is inventor on several patents related to the PMCA technology and is currently Founder, Chief Scientific Officer and Vice-President of Amprion Inc, a biotech company focusing on the commercial utilization of PMCA for prion diagnosis.

Table 1: Summary of the blood samples analyzed and the results obtained in PrP^{Sc} detection by PMCA

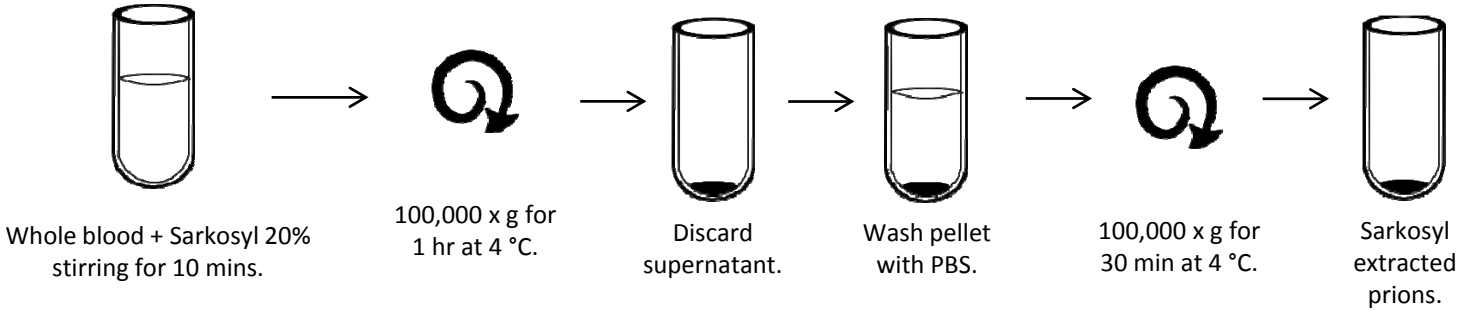
Clinical diagnosis	**Total patients**	**PrP^{Sc} detected in blood**
Variant Creutzfeldt-Jakob disease	14	14/14
Sporadic Creutzfeldt-Jakob disease[a]	16	0/16
Other neurodegenerative diseases[b]	60	0/60
Other neurological diseases[c]	26	0/26
Healthy controls	49	0/49

[a] Of these 16 sCJD samples analyzed, 6 were whole blood, 5 plasma and 5 white blood cells from distinct sCJD patients.

[b] Includes samples from patients with Alzheimer's disease, Parkinson's disease, Lewy Body dementia, and fronto-temporal dementia

[c] Include samples from patients with vascular dementia, seizures, epilepsy, psychiatric diseases, traumatic brain injury, mild cognitive impairment, demyelinating disease and encephalitis.

Figure 1


Whole blood + Sarkosyl 20% stirring for 10 mins.
100,000 x g for 1 hr at 4 °C.
Discard supernatant.
Wash pellet with PBS.
100,000 x g for 30 min at 4 °C.
Sarkosyl extracted prions.

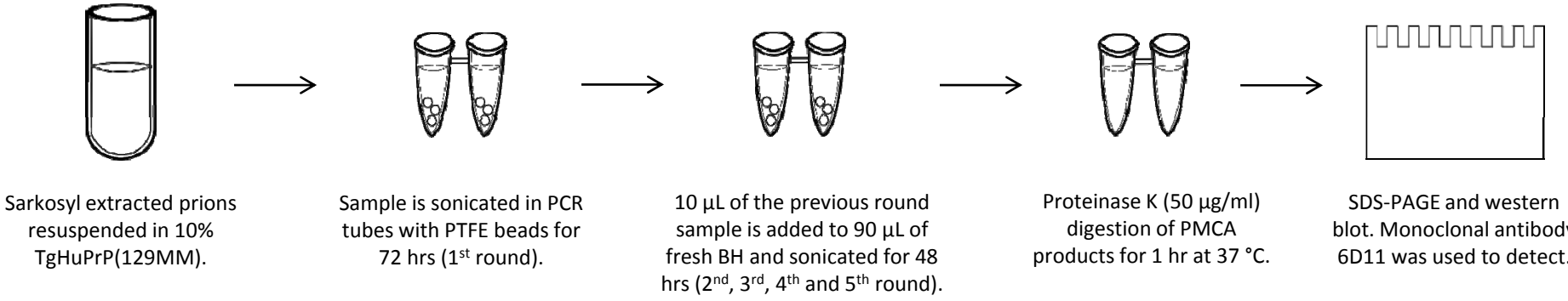

Sarkosyl extracted prions resuspended in 10% TgHuPrP(129MM).
Sample is sonicated in PCR tubes with PTFE beads for 72 hrs (1st round).
10 µL of the previous round sample is added to 90 µL of fresh BH and sonicated for 48 hrs (2nd, 3rd, 4th and 5th round).
Proteinase K (50 µg/ml) digestion of PMCA products for 1 hr at 37 °C.
SDS-PAGE and western blot. Monoclonal antibody 6D11 was used to detect.

Figure 2

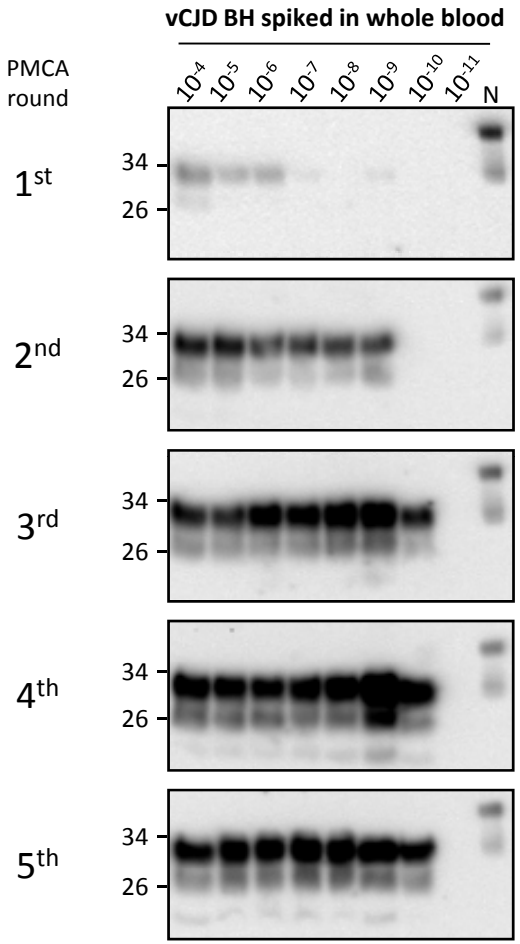

vCJD BH spiked in whole blood
PMCA round
10-4
10-5
10-6
10-7
10-8
10-9
10-10
10-11
N
1st
2nd
3rd
4th
5th
34
26

Figure 3

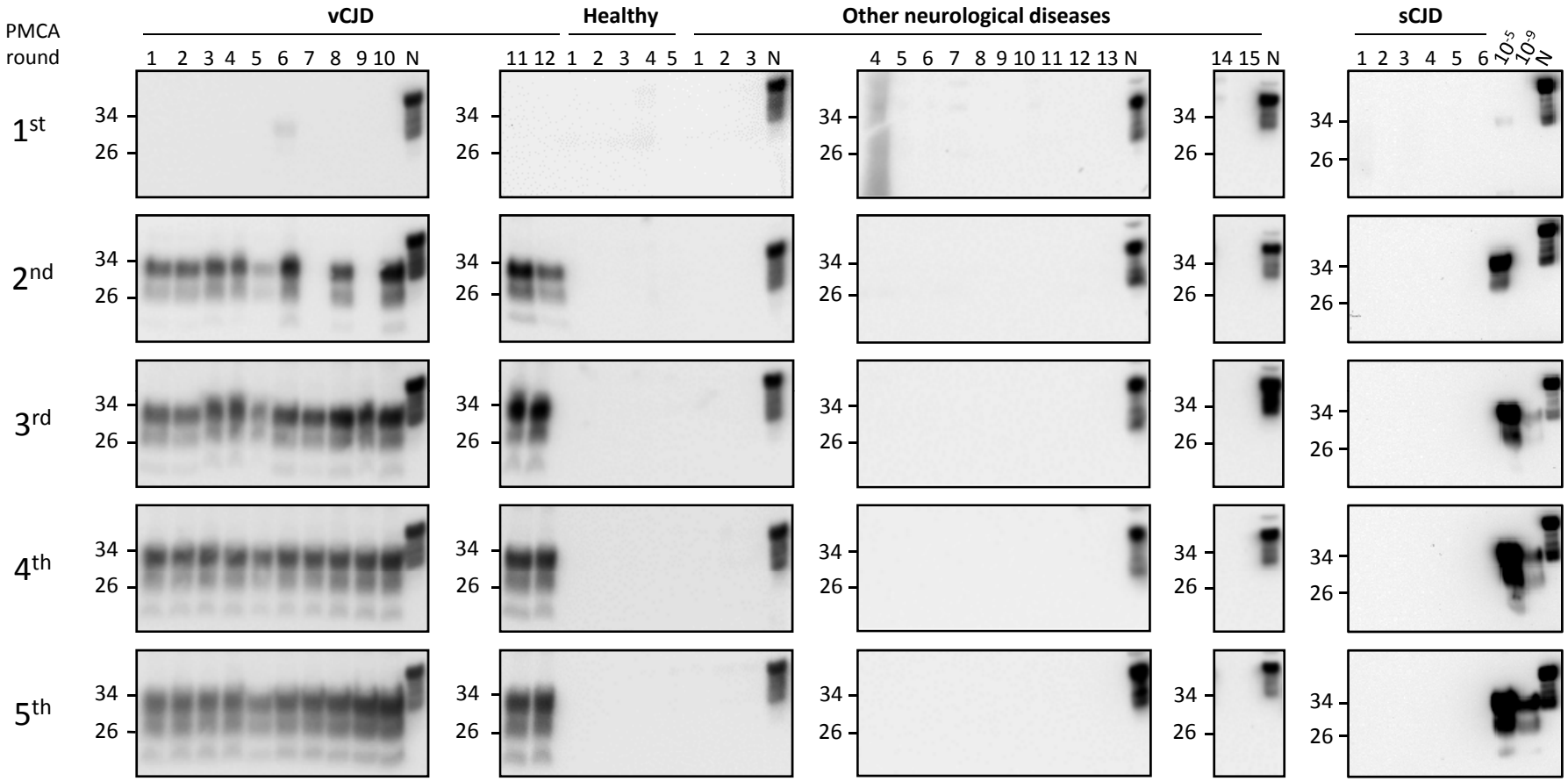

PMCA round
vCJD
Healthy
Other neurological diseases
sCJD
1 2 3 4 5 6 7 8 9 10 N
11 12 1 2 3 4 5
1 2 3 N
4 5 6 7 8 9 10 11 12 13 N
14 15 N
1 2 3 4 5 6 10-5 10-9 N
1st
2nd
3rd
4th
5th
34
26

Figure 4

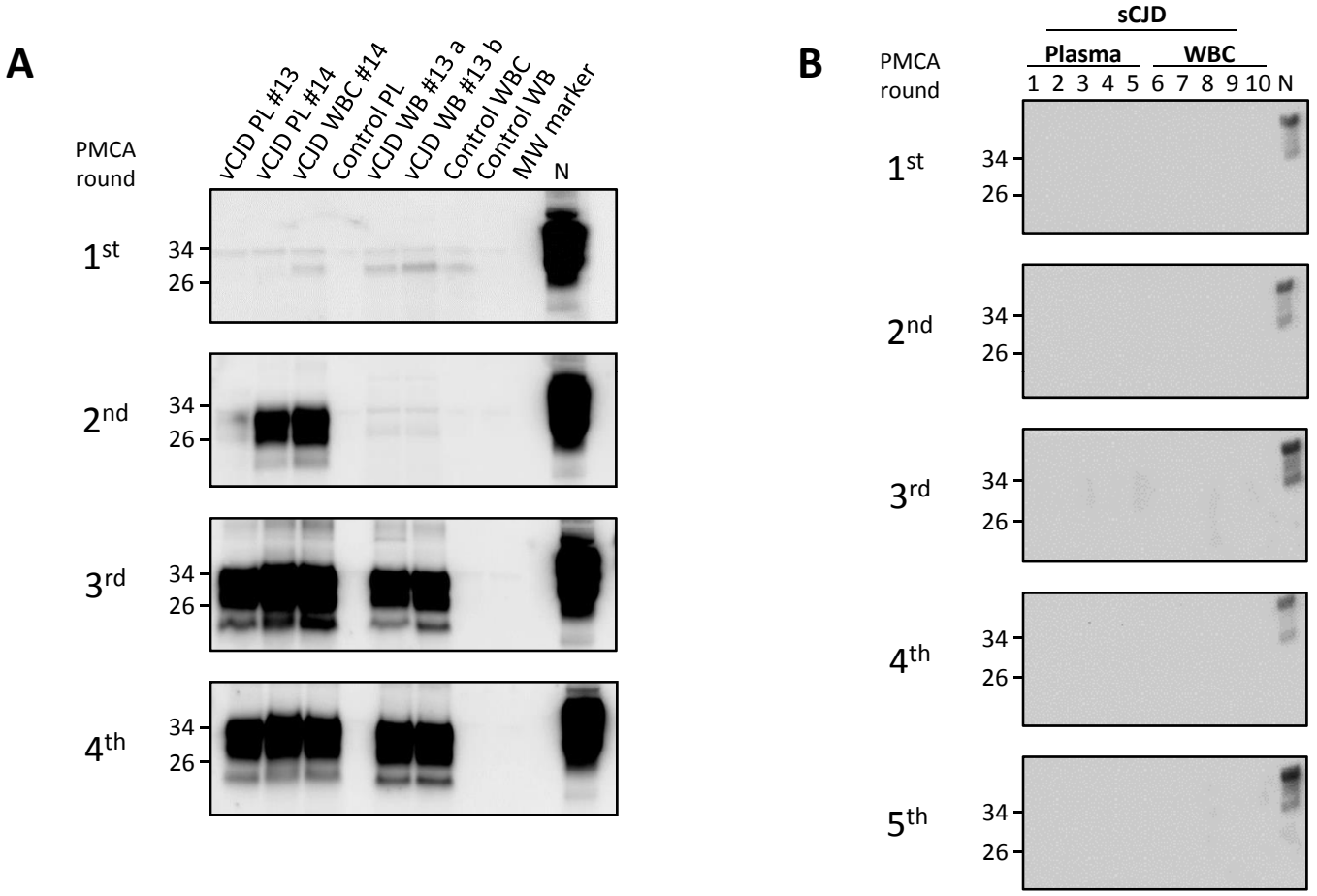
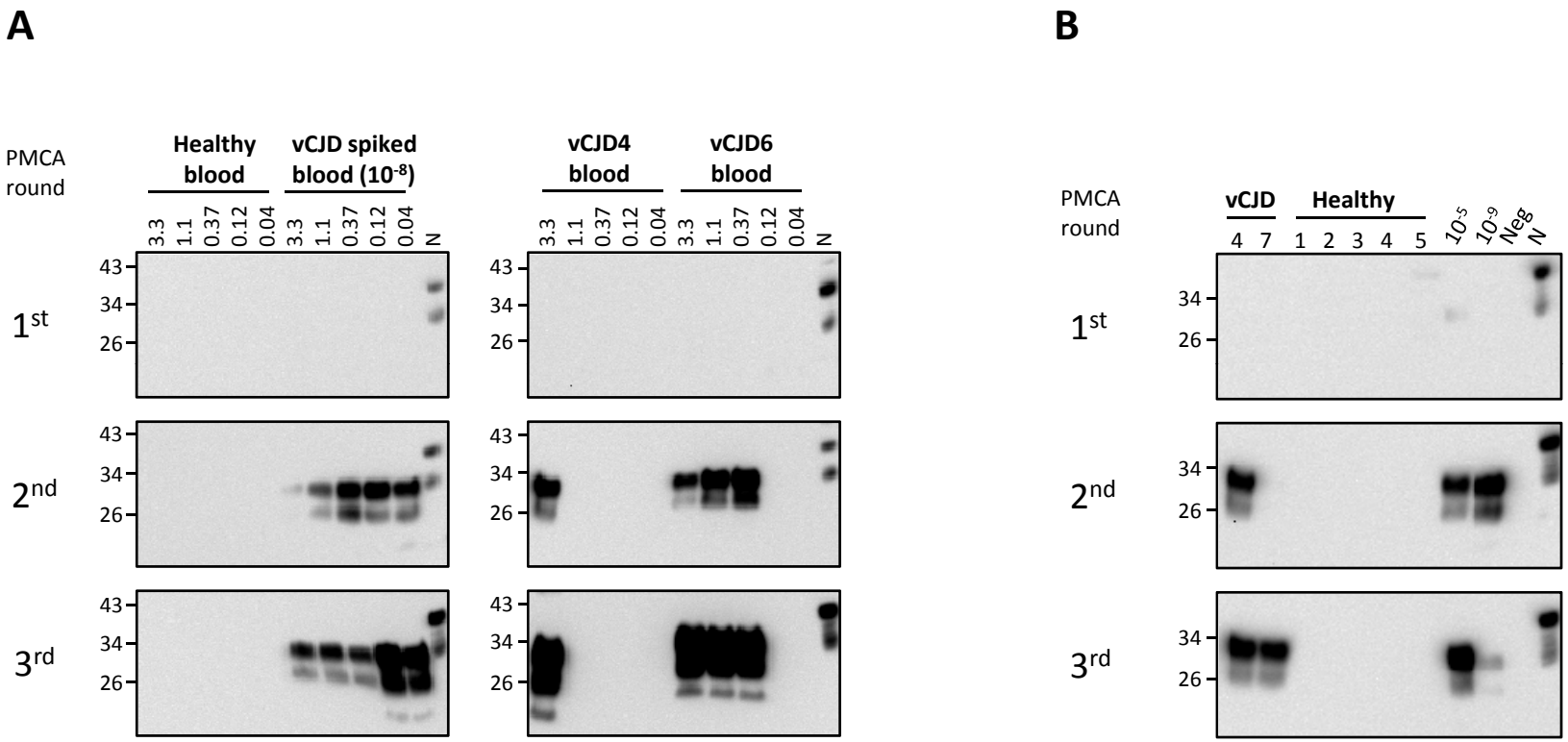

A
PMCA round
vCJD PL #13
vCJD PL #14
vCJD WBC #14
Control PL
vCJD WB #13 a
vCJD WB #13 b
Control WBC
Control WB
MW marker
N
1st
2nd
3rd
4th
34
26
B
sCJD
Plasma
WBC
1 2 3 4 5 6 7 8 9 10 N
PMCA round
1st
2nd
3rd
4th
5th
34
26

Figure 5


A
PMCA round
Healthy blood
vCJD spiked blood (10^{-8})
vCJD4 blood
vCJD6 blood
3.3 1.1 0.37 0.12 0.04 3.3 1.1 0.37 0.12 0.04 N
3.3 1.1 0.37 0.12 0.04 3.3 1.1 0.37 0.12 0.04 N
1st
2nd
3rd
43
34
26
B
PMCA round
vCJD
Healthy
4 7 1 2 3 4 5 10^{-5} 10^{-9} Neg N
1st
2nd
3rd
34
26